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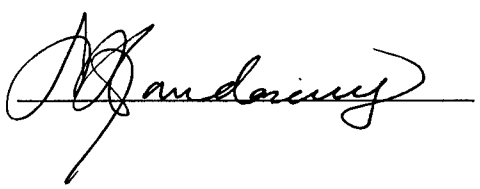

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13. ABSTRACT ***(Maximum 200 Words)***

This research is designed to test the hypothesis that aneuploidy in some breast tumors is caused by centrosome abnormalities which are induced by alteration in p53 function. Specific mutations in p53 that are associated with breast cancer, aneuploidy, and centrosome abnormalities have been identified during the course of this project. We have shown that p53 mutations correlate with an increase in microtubule nucleating capacity; however, no such correlation is indicated with either centrosome size or centrosome number. To test whether or not a specific p53 mutation affects centrosome function, thus leading to chromosomal instability, attempts are being made to transfect normal mammary breast cells to over-express selected mutants of p53. Once transfected, the cells will be monitored for changes in ploidy and centrosome structure and function. Due to the poor transfection efficiency using standard plasmid-based transfections with a number of different lipid transfection reagents, we have recently begun to construct adenoviral vectors for our mutant p53 studies. The high efficiency of transduction with these adenoviral vectors will allow us to perform our planned studies.

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INTRODUCTION

This research is designed to test the hypothesis that aneuploidy in some breast tumors is caused by centrosome abnormalities which are induced by alteration in p53 function. Specific mutations in p53 that are associated with breast cancer, aneuploidy, and centrosome abnormalities have been identified during the course of this project. We have shown that p53 mutations correlate with an increase in microtubule nucleating capacity; however, no such correlation is indicated with either centrosome size or centrosome number. To test whether or not a specific p53 mutation affects centrosome function, thus leading to chromosomal instability, attempts are being made to transfect normal mammary breast cells to over-express selected mutants of p53. Once transfected, the cells will be monitored for changes in ploidy and centrosome structure and function. Due to the poor transfection efficiency using standard plasmid-based transfections with a number of different lipid transfection reagents, we have recently begun to construct adenoviral vectors for our mutant p53 studies. The high efficiency of transduction with these adenoviral vectors will allow us to perform our planned studies.

BODY

As indicated in 1999 Progress Report, I revised my statement of work in order to use a new Mayo Facility for the p53 mutation screening portion of the project. Research Accomplishments to date reflect the Tasks as outlined in the revised Statement of Work.

Task 1 - Quantification of structural and functional centrosome alterations (months 1-12). As reported in the 2000 Progress Report, this Task is complete. Some of the results from this task were published in American Journal of Pathology (155:1941-1951), a reprint of which was appended to the 2000 Progress Report.

Task 2 – Screen tissues for aneuploidy (months 10-18). As reported in the 2000 Progress Report, approximately 35 benign and tumor tissues have been analyzed for ploidy using FISH analysis of chromosomes 3,7,and 17. The methods used were changed from the original proposal to yield specific information on chromosome 17, which is the location of the p53 gene. This Task is complete and data analysis is near completion. Thus far, the analysis reveals that: 1) all benign tissues were diploid and had normal centrosomes; 2) three of 21 tumors were diploid or near diploid and had essentially normal centrosome size and function; and 3) 18 of 21 tumors were aneuploid and had significant levels of centrosome amplification. **Therefore, we can conclude that tumor aneuploidy correlates with centrosome amplification.** During the past year a manuscript has been written which includes a portion of these results. This manuscript has been submitted to Cancer Research for publication (Appendix 1). **Further analysis of the data has revealed that, regardless of p53 status, centrosome number and centrosome size each have a statistically significant linear correlation with chromosomal instability. However, centrosome function, as measured by microtubule nucleation capacity, does not correlate with chromosomal instability. Interestingly, microtubule nucleation capacity correlates with loss of differentiation as measured by Nottingham grade.** These results will be presented in a manuscript that is currently in preparation (see Appendix 2 for the abstract of this manuscript).

Task 3 – Trial site-directed mutagenesis and trial transfection. A p53 mutated from glycine to serine at amino acid 245 was selected based on its occurrence in Li-Fraumeni families having a high incidence of

breast cancers. In last year's Progress Report, I stated that "**Initial results indicated that cells transfected with the p53 mutant develop a phenotype consistent with the hypothesis, namely centrosome and mitotic spindle abnormalities are present at a much higher frequency in the presence of mutant p53 than they are in normal cells.** Because transfected primary mammary epithelial cells have proven too difficult to work with, we are now using primary mammary epithelial cells that have been transfected with human telomerase for these studies. These cells (hTERT-hMECs) were obtained from Geron, Inc. under a Materials Transfer Agreement." This Task has proven to be the most challenging component of the research. We are still having difficulties in achieving consistently high transfection efficiencies. We have tried numerous transfection reagents and conditions, with poor success. Unfortunately, very little further progress has been made on this task during the last year. In order to complete this task and task 6, we are currently making adenoviral constructs of the G245S p53 mutant. We tested the adenovirus for possible toxicity and for the potential for infection of normal human mammary epithelial cells (hMEC) and hTERT-hMEC cells using a GFP reporter construct. **Virtually 100% of hTERT-hMEC cells were infected with no signs of toxicity after 72 hours using intermediate and high multiplicity of infection (MOI).** At the highest MOI used, virtually 100% of hMEC cells were infected, but there were some toxic effects. At intermediate MOI, 60-80% of the cells were infected and there were no signs of toxicity. Tighter titration of the MOI should allow us to achieve our goal of 80% infection while minimizing toxicity. We are at the stage of plaque selection for our first mutant vector. Using adenoviral vectors to deliver the mutant p53 cDNA will allow us to complete Tasks 3 and 6.

Task 4 – p53 mutation/immunohistochemistry status (months 16-30). As reported last year, all tissues have been analyzed for p53 immunohistochemistry. Normal tissues had no significant p53 immunostaining. Benign tumors had an average value of 3.6% of the cells with p53 immunostaining (ranging from 0 to 10% of the tumor cells). Malignant tumors ranged from 0% (13 of 40 analyzed) to more than 75% (8 of 40 analyzed), with a mean of 13.8%. During the past year mutation screening of exons 4-9 was performed using DHPLC after DNA extraction and PCR amplification of 34 tumors. Sequence confirmation of mutations identified by DHPLC is near completion. Some of these data are presented in a manuscript that has been submitted for publication in Cancer Research (Appendix 1). In summary, we found that 21 tumors had wild type p53 by DHPLC. The 13 tumors indicated by DHPLC as containing possible p53 mutations have been sequenced and the precise mutation has been identified in 8 of the tumors. One of the eight mutations (in exon 6) was silent. The other 7 mutations resulted in changes in the amino acid sequence of p53 in the DNA binding domain. Exons 5, 7, and 8 each had 2 tumors containing mutations, while 1 tumor had a mutation in exon 6. One of the mutations is the commonly occurring R249G mutation; no mutations have been found in exons 4 or 9. Mutations have not been identified in 5 tumors; these are being sequenced again to determine the mutation or to verify the absence of a mutation. This task is nearly complete.

Task 5 – Analysis of data from Tasks 1,2, and 4 (months 31-33). A preliminary analysis comparing the tumors containing documented p53 mutation with tumors containing wild type p53 reveals that the centrosome microtubule nucleating capacity in p53 mutant tumors is 2 fold higher than in p53 wild type tumors; this difference is statistically significant. In our small sample size we were unable to detect differences in other measures of centrosome amplification. On average, tumors with mutant p53 did not have centrosomes that differed significantly in either size or number from those with wild type p53. Never-the-less, we have identified a series of three p53 mutations (described in Task 6) that do appear to have significant effects on centrosome structure and function. The first of these mutants is a termination mutant at amino acid 195. The tumor with this mutation has a high level of chromosomal instability and

numerous, large centrosomes per cell. However, it has only a moderate increase in microtubule nucleation capacity. The second mutation is R249G, a commonly mutated codon in many cancers. The tumor with this mutation has centrosomes highly amplified by all measures and a significant level of chromosomal instability. The third mutation, C238F, is in a tumor that is nearly normal in centrosome size and number, but has the highest microtubule nucleation capacity of all the tumors we measured. This tumor has a stable, but aneuploid, karyotype. As we complete the sequence confirmation of the other 5 possible mutants in Task 4, we may identify more mutations to pursue with further studies.

Task 6 – Site-directed mutagenesis of p53 using mutants identified in Task 5 (months 33- 36). We have begun creating cDNAs by site-directed mutagenesis for the three p53 mutations identified in Task 5. These mutations are: 1) R196stop, in which a mutation of C to T at base 586 in exon 6 codes for STOP instead of Arginine, truncating the protein at 195 amino acids; 2) C238F, in which a G to T mutation at base 713 in exon 7 codes for phenylalanine instead of cysteine at codon 238; and 3) R249G, in which mutation of A to G at base 745 in exon 7 codes for glycine instead of arginine at amino acid 249. Site-directed mutagenesis is in progress prior to creation of adenoviral vectors containing these mutated p53 cDNAs.

Task 7 – Transfection and monitoring experiments (months 35-46). Not yet begun. The first adenoviral vectors for these experiments are expected to be ready for use beginning in October 2001.

Task 8 – Data analysis and manuscript preparation (months 38-48). As reported last year, the first paper resulting from this project has been published in American Journal of Pathology (155:1941-1951). Portions of the data from Tasks 2 and 4 have been incorporated into a manuscript that has been submitted to Cancer Research for publication (Appendix 1). Another manuscript presenting data from Tasks 2 and 4 is in preparation (see Abstract in Appendix 2).

KEY RESEARCH ACCOMPLISHMENTS

- Excess pericentriolar material is a specific centrosome defect associated with an increased frequency of abnormal mitoses in human breast tumors.
- A specific p53 mutation (glycine to serine at amino acid 245) induces abnormal centrosome structure and function upon transfection of primary normal human mammary epithelial cells.
- **Tumor aneuploidy correlates with centrosome amplification.**
- **Centrosome number and centrosome size each have a statistically significant linear correlation with chromosomal instability.**
- **Centrosome microtubule nucleation capacity does not correlate with chromosomal instability.**
- **Centrosome microtubule nucleation capacity does correlate with loss of tumor differentiation.**
- **Tumors with p53 mutations have a statistically significant 2 fold higher capacity to nucleate microtubules than tumors with wild type p53.**
- **p53 mutations have been identified in breast tumors that are associated with centrosome amplification and aneuploidy.**

REPORTABLE OUTCOMES

Invited Seminars

1. Aberrant Structure and Function of Centrosomes in Human Breast Tumors. March 1999 GI Unit Scientific Meeting, Mayo Clinic, Rochester, MN.
2. Breast Cancer: Centrosomes, Aneuploidy, and Progression. February 2000. University of Puerto Rico Medical School, San Juan, PR.
3. Aberrant Structure and Function of Centrosomes in Breast Cancer. June 2000. Department of Cellular Biology, University of Georgia, Athens, GA.
4. Centrosomes, Aneuploidy, and Breast Cancer. 2000. Mayo Laboratory Society, Mayo Clinic, Rochester, MN.
5. Chromosomal Instability Correlates with Centrosome Amplification in Human Breast Cancer. June 2001. GI Research Seminar Series. Mayo Clinic, Rochester, MN.
6. The Role of the Centrosome in Development and Progression of Breast Cancer. August 2001. Invited Speaker in "Biology of Cancer" Symposium at the Annual Meeting of the Microscopy Society of America. Long Beach, California.

Publications

1. Salisbury, JL, Whitehead, CM, **Lingle, WL**, and Barrett, SL. 1999. Centrosomes and cancer. Biology of the Cell. 91:451-460.
2. Salisbury, JL, **Lingle, WL,** White, RA, Cordes, LEM, and Barrett, SL. 1999. A simple method to assess microtubule nucleating capacity of centrosomes in tissue sections. J. Histochem. Cytochem. 47:1265-1273.
3. **Lingle, WL** and Salisbury, JL. 1999. Altered centrosome structure in human breast tumors. American J. Pathol. 155:1941-1951. **Cover photo.**
4. **Lingle, WL** and Salisbury, JL. 2000. The role of the centrosome in the development of malignant tumors. In: The centrosome in reproduction and cell replication (Palazzo, RE, ed). Current Topics in Developmental Biology, Academic Press. 49:313-329.
5. Lutz, W, **Lingle, WL**, McCormick, D, Greenwood, TM, and Salisbury, JL. 2001. Phosphorylation of centrin during the cell cycle and its role in centriole separation preceding centrosome duplication. J. Biological Chemistry. 276:20774-20780.
6. **Lingle, WL** and Salisbury, JL. 2001. Microtubule nucleation assay to measure centrosome amplification in tissues. In: Methods in Cell Biology, Centrosome in Cell Replication and Early Development (Palazzo, RE, ed). Academic Press. In Press.

Manuscripts Submitted

1. Antonino B. D'Assoro, Susan L. Barrett, Christopher Folk, Vivian C. Negron, Kelly Boeneman, Robert Busby, Clark Whitehead, Franca Stivala, **Wilma L. Lingle** and Jeffrey L. Salisbury. Centrosome Amplification Marks the Transition Leading to More Aggressive Phenotypes in Breast Cancer. Submitted to Cancer Research, June 15, 2001.

Manuscripts in Preparation

1. Salisbury, JL, D'Assoro, A, Whitehead, CM, and **WL Lingle**. Centrosome amplification correlates with chromosomal instability in human breast tumors. To be submitted to Cancer Research, July 31, 2001.

Research Funding Received On The Basis Of Work Supported By This Award

DAMD 17-01-1-0753 (**Lingle**, PI) 8/1/01 – 7/31/04 25%
DoD Breast Cancer Research Program $391,521
"Investigation of gene expression correlating with centrosome amplification in development and progression of breast cancer"

Eagles' Award (**Lingle**, PI) 07/01/01 – 06/30/02
Mayo Clinic Internal Cancer Research Program $30,000
"Effects of Mutant p53 on the Structure and Function of Centrosomes and the Mitotic Spindle Apparatus"

Research Funding Applied For On The Basis Of Work Supported By This Award

Idea Award (Couch, PI; **Lingle**, Co-Investigator) 8/01/02 – 7/31/04 10%
DoD Breast Cancer Research Program $444,842
"The Role of the δ-Tubulin Oncogene in Breast Cancer"

Breast Cancer Center of Excellence Award 1/02 - 12/05 10%
(Hartmann, PI; **Lingle**, collaborator)
DoD Breast Cancer Research Program $4,000,000
"Benign Breast Disease: Toward the Molecular Prediction of Breast Cancer Risk"

Promotion received on the basis of experience supported by this award
Senior Research Fellow to Associate Consultant, effective March 1, 1999.

CONCLUSIONS

To date, the research supported by this award has yielded results with significant implications regarding the origin and perpetuation of aneuploidy in breast cancer, especially related to centrosome amplification and p53 mutation. First, excess pericentriolar material has been linked with an increased frequency of abnormal mitoses in tumor tissues. This demonstrates that at least one aspect of centrosome amplification is associated with mitotic events that most often result in aneuploid daughter cells. Furthermore, the increase in amplified centrosomes and abnormal mitoses was duplicated in limited *in vitro* studies. In these studies, cultured normal mammary epithelial cells were transfected with G245S mutant p53; indicating that **p53 mutation may be involved in centrosome amplification associated with abnormal mitoses that can lead to aneuploidy.**

A second important result demonstrated the correlation of aneuploidy with centrosome amplification in breast tumors using fluorescence in situ hybridization with centromeric probes to chromosomes 3, 7, and 17. In these studies, 18 of 21 tumors were found to be aneuploid while three were diploid. The three diploid tumors had nearly normal centrosomes, while all 18 aneuploid tumors displayed centrosome amplification. **Further analysis revealed a statistically significant linear correlation between chromosomal instability and centrosome number and centrosome size. However, there was no correlation between the microtubule nucleation capacity of tumor centrosomes and chromosomal instability.** Microtubule nucleation capacity of tumors does show a correlation with loss of tissue differentiation as determined by the Nottingham Grading system. The less differentiated Grade II and

III tumors had significantly greater microtubule nucleation capacity than the more differentiated Grade I tumors, and all grades were significantly greater than fully differentiated normal tissues. **Together, these results indicate that different aspects of centrosome amplification have different effects on tumor progression. Centrosome size and number affect chromosomal instability, while microtubule nucleation capacity affects cell, and therefore, tissue architecture. Chromosome instability has profound implications for tumor progression, while changes in cell architecture can increase metastatic potential.**

Third, on average, centrosome of tumors with p53 mutations had a 2 fold greater capacity to nucleate microtubules than centrosomes from tumors with wild type p53. The two other measures of centrosome amplification used in this study, centrosome size and centrosome number, were not significantly different between the two groups. **In light of the conclusions from the previous paragraph, this means that mutations in p53 may increase tumor grade by affecting the microtubule cytoskeleton of the tumor cells.**

APPENDICES

Appendix 1 – Manuscripts Submitted

1. Antonino B. D'Assoro, Susan L. Barrett, Christopher Folk, Vivian C. Negron, Kelly Boeneman, Robert Busby, Clark Whitehead, Franca Stivala, **Wilma L. Lingle** and Jeffrey L. Salisbury. Centrosome Amplification Marks the Transition Leading to More Aggressive Phenotypes in Breast Cancer. Submitted to Cancer Research, June 15, 2001.

Appendix 2 - Manuscripts in Preparation

2. Salisbury, JL, D'Assoro, A, Whitehead, CM, and **WL Lingle**. Centrosome amplification correlates with chromosomal instability in human breast tumors. To be submitted to Cancer Research, July 31, 2001.

Centrosome Amplification Marks The Transition Leading To More Aggressive Phenotypes In Breast Cancer

Antonino B. D'Assoro[1], Susan L. Barrett[1], Christopher Folk[1], Vivian C. Negron[1], Kelly Boeneman[1], Robert Busby[1], Clark Whitehead[1], Franca Stivala[4], Wilma L. Lingle[1, 2,3] and Jeffrey L. Salisbury[1,3]

[1]Tumor Biology Program, [2]Experimental Pathology, Mayo Clinic Foundation, Rochester, MN 55905 and [4] Institute of General Pathology, University of Catania, Italy 95123

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Running title: **Centrosome Amplification in Breast Cancer**

Key Words: estrogen receptor, p53, p21/waf1, aneuploidy, mitotic spindle

ABSTRACT

Molecular mechanisms leading to genomic instability and phenotypic variation during tumor development and progression are poorly understood. Centrosome amplification drives the development of chromosomal instability and genetic heterogeneity in solid tumors by leading to multipolar mitoses and unequal chromosome segregation. Such instability represents a major problem in the management of breast cancer because of its contribution to more aggressive phenotypes as well as chemoresistence. In this study we analyzed breast carcinomas and tumor-derived cell lines to determine the relationship between centrosome amplification and established prognostic factors. Our results suggest that centrosome amplification is a common feature of aneuploid breast tumors and could mark the transition to more aggressive phenotypes. Based on these observations we propose that determination of centrosome defects may be useful as an additional prognostic marker and in further stratifying patients with hormone responsive and lymph node negative phenotypes for adjuvant chemotherapy.

INTRODUCTION

Breast cancer, like most other cancers, is characterized by complex and heterogeneous genetic alterations originating from genomic instability and aneuploidy (1). Genomic instability leads to the persistent generation of new chromosomal variations, to tumor progression and to the development of more aggressive phenotypes with increased metastatic potential and chemoresistence (2). The steroid hormone estradiol plays an important role in the etiology of breast cancer (3). Often, breast tumors progress from a hormone-dependent to a more aggressive hormone-independent phenotype (4). Hormone-independent tumors are less likely to be well differentiated, are aneuploid and in general show more frequent mutations, including loss or amplification of breast cancer related genes (p53, ErbB2/HER-2/neu, EGFR) (5). For this reason, the hormone responsive status of breast tumors is considered one of the most important prognostic factors for predicting clinical outcome, and so determines appropriate treatment for breast cancer patients.

Centrosome defects are characteristic of breast cancer and solid tumors in general (6-11). The centrosome plays an essential role in equal segregation of chromosomes through the establishment of the bipolar mitotic spindle. Precise control of centrosome duplication is strictly coordinated with DNA replication during cell cycle progression. In cancer cells, alteration of centrosome homeostasis by disregulation of cell cycle checkpoints leads to centriole over-duplication and multipolar mitoses, thereby increasing the rate of chromosomal instability. Interestingly, loss of function of the tumor suppressor gene p53 leads to centrosome amplification and aneuploidy (7, 12-15). The mechanism for p53 control of centrosome duplication is probably mediated, in part, through transcriptional regulation of the cyclin-dependent kinase inhibitor p21/*Waf1* and its subsequent inhibition of Cdk/cyclin D and E activity (16-19).

In this study we analyzed centrosome characteristics in breast carcinomas and tumor derived cell lines with different phenotypes to clarify the relationship between centrosome amplification and estrogen receptor (ER), ErbB2, EGFR and p21/*Waf1* expression, p53 status, mitotic abnormalities and metastatic potential. Our studies demonstrate that centrosome amplification is linked to chromosomal instability and tumor aggressiveness. Based on these observations we propose that centrosome amplification could play a key role in breast cancer progression through the development of phenotypic variation and that this amplification may be useful as an additional prognostic marker and/or novel target for clinical intervention in breast cancer.

MATERIALS AND METHODS

Human tissue samples and cell culture. Human breast tissue was obtained immediately after surgery according to an IRB approved protocol, frozen in liquid nitrogen and stored at –70 C until use. For electron microscopy fresh tissue was processed immediately in Trumps fixative according to (20). Specimens were obtained from patients who had no chemotherapeutic or radiation treatments before surgery. Human breast cancer cell lines were obtained from ATCC. MCF-7 and T-47D cells were grown in RPMI 1640 medium,

and MDA-MB231 and MDA-MB435 cells were grown in MEM medium supplemented with 2mM L-glutamine, 1% penicillin/streptomycin and 10% fetal bovine serum in 5% CO_2 in air at 37 C.

Microtubule nucleation assay. Breast cancer cell lines grown on glass cover slips and cryosections of breast tissue were processed as described earlier (21). Microtubule nucleation capacity is reported as the *MT Index* = average # microtubules nucleated by the experimental specimen (n=50) divided by the average # microtubules nucleated by the control specimen (n=50). For MT Index of cell lines, normal human mammary epithelial cell cultures (HMEC, Clonetics, Walkersville, MD) were used as controls. The MT Index for tissues was determined against the average value for 5 separate normal mammary epithelial tissue specimens set as the normalized control value.

Microscopy and Western analyses. Antibodies used for indirect immunofluorescence and Western analysis were obtained from the following sources: β-actin, α-tubulin, γ-tubulin, ER (Sigma, St. Louis, MO), pericentrin (Babco, Richmond, CA), p53 (DAKO, Mississauga, ON, Canada), ErbB2 (Santa Cruz, Santa Cruz, CA), EGFR (Genosys, The Woodland, TX), and p21/*Waf1* (Oncogene, Boston, MA), centrin (20H5, our laboratory), and HsEg5 (a generous gift from Dr. JB Rattner (22)). For indirect immunofluorescence the primary antibodies were followed by FITC, Alexa 488, Alexa 568 (Molecular Probes, Eugene, OR), or rhodamine-conjugated secondary antibodies and Hoechst 33342 stain for DNA.

Methods for indirect immunofluorescence and Western analysis were carried out as described earlier (9). Samples were observed by using a Nikon FXA epifluorescence microscope or a Zeiss LSM 510 scanning laser confocal microscope, and images were recorded electronically. Spindle morphology was scored as normal 'bipolar' if fewer than 1% aberrant spindles were present (n = 100 spindles). Mitotic figures in the normal breast tissue were extremely rare, however in the several instances where they were found they were bipolar. For Western blots, cell lysates (50 μg) were separated by SDS/PAGE, transferred to PVDF membranes (Millipore, Bedford, MA), reacted with

appropriate antibody followed by HRP-conjugated secondary, and visualized by chemiluminescence using ECL reagents according to manufacturer's instructions (Amersham, Piscataway, NJ).

Fluorescent *in situ* hybridization (FISH). A custom mixture of probes for human chromosomes 3 (red), 7 (green), and 17 (aqua) obtained from Vysis, Inc. (Downers Grove, Il) was hybridized to 'touch' preparations of human breast tissue according to published methods (23). One hundred nuclei were scored for each tumor specimen.

Analysis of p53 and estrogen receptor status. In breast tissue p53 status was determined by denaturing high performance liquid chromatography of PCR products (24) for detection of mutations in exons 4-9 and confirmed by subsequent sequencing for all tumor samples. Estrogen receptor status was determined using an immunohistochemistry-based assay (25).

RESULTS

Centrosome phenotypes, estrogen dependence, p53 status and metastatic potential in breast cancer cell lines. For *in vitro* studies, we used four human breast cancer cell lines (MCF-7, T-47D, MDA-MB231, and MDA-MB435) to investigate the relationship between centrosome amplification and established prognostic markers of breast cancer. Centrosome amplification was assessed by both structural and functional criteria. For these studies the number of centrioles and the amount of the surrounding pericentriolar material (PCM) were determined using immunofluorescence for centrin and pericentrin, respectively. Centrosome function was determined using a quantitative assay for microtubule nucleation and mitotic spindle morphology. The four cell lines were ordered according to increasing centrosome amplification from normal to highly amplified (Fig. 1). The breast tumor cell line MCF-7 showed normal appearing centrosomes with two or four centrin staining spots (centrioles) surrounded by a moderate amount of PCM (Fig. 1 a, b, Table I). In contrast, the breast tumor cell lines T-47D, MDA-MB231 and MDA-MB435 showed increased centrosome amplification characterized by multiple centrin

staining spots (supernumerary centrioles) and excess PCM (Fig 1 a, b, Table I). Since tumor centrosomes are typified by alterations in microtubule nucleation (8, 9), we performed a functional assay to determine microtubule nucleation capacity on detergent extracted models of the four breast cancer cell lines. These studies revealed that increased levels of microtubule nucleation corresponded to centrosome amplification (Fig. 1 c and Table I). Similarly, the four cell lines displayed a range of mitotic spindle abnormalities (Fig. 1 d and Table I). Each of the cell lines showed the majority of dividing cells with normal mitotic figures. Nonetheless, MCF-7 cells showed 10% abnormal mitoses, and T-47D, MDA-MB231 and MDA-MB435 showed a higher frequency (14-22%) of aberrant mitoses, including multipolar spindles, lagging chromosomes and cytokinesis defects.

These same four human breast tumor cell lines were assessed for ER, ErbB2, EGFR, and p21/*Waf1* expression and p53 mutation status. The estrogen-dependent cell lines MCF-7 and T-47D both show ER expression by Western analysis, while the estrogen-independent cell lines MDA-MB231 and MDA-MB435 lack ER entirely (Fig. 2). As described earlier (26), each of these cell lines showed a distinctive growth factor receptor expression pattern. ErbB2 expression was found to be highest in T-47D, nominal in MCF-7 and MDA-MB231, and lowest in the MDA-MB435 cell line. In contrast, EGFR expression was high in the MDA-MB231, low in T-47D, and undetectable in MCF-7 or MDA-MB435 cell lines (Fig. 2). MCF-7, with a wild-type p53 phenotype (27), showed no detectable p53 (Figs. 1 e and 2), whereas T-47D, MDA-MB231 and MDA-MB435 cell lines, which have been reported to express mutant p53, showed high levels of p53 accumulation that was largely localized in the nucleus (Figs. 1 e and 2). Expression and localization studies also showed a high level of expression of the cdk/cyclin inhibitor p21/*Waf1*, a downstream effector of the p53 pathway, in MCF-7 and T-47D, and low levels in MDA-MB231 and MDA-MB435 cells (Figs. 1 f and 2). The metastatic potential of the four cell lines has been determined in nude mice (28, 29), and in this model system MCF-7 shows the least aggressive behavior, with increasing metastatic potential observed for T-47D, MDA-MD231 and MDA-MD435, respectively. Similarly, the degree of genomic instability as measured by chromosomal instability increases from

the MCF-7 to more variable T-47D, MDA-MD231 and MDA-MD435 cell lines (30, 31). Table I summarizes the characteristics of the four breast cancer cell lines in relation to centrosome phenotype. Taken together, these results demonstrate that centrosome amplification increases with loss of estrogen-dependence and p53 function, and with demonstrated metastatic potential and genomic instability.

Centrosome amplification and chromosomal instability in breast tumors. For studies on human breast tissue we analyzed five normal and twenty-one invasive breast tumor specimens for centrosome amplification, aberrant mitoses, DNA ploidy, ER, p53 and lymph node status, and metastasis (Fig. 3 and Table I). We selected five breast tumors with distinct phenotypes, three of which matched the phenotypes of the breast cancer cell lines described above. Like the human breast cancer cell lines, these tumors could be arranged according to increasing centrosome amplification based on centrin and pericentrin staining (Fig. 3 a-e). In these breast tumors, the level of centrosome amplification also reflected the degree of aneuploidy based on FISH analysis of chromosomes 3, 7 and 17, where the diploid tumor showed nearly normal centrosomes and aneuploid tumors showed centrosome amplification (Fig. 3). Interestingly, several important findings emerged from these studies. Diploid breast tumors consistently displayed normal centrosomes, bipolar mitotic spindle morphology, and were positive for ER, whereas all aneuploid tumors showed amplified centrosomes and higher levels of mitotic abnormalities (Fig. 3 and Table I). Finally, the most severe examples of centrosome amplification occurred in more aggressive tumor phenotypes based on lymph node status and frank metastasis (Fig. 3 d-e and Table I). These observations confirm the correlation between centrosome amplification with an increase in mitotic abnormalities (9, 20)and demonstrate a relationship with aneuploidy, loss of ER expression and p53 function (p53 mutation) and tumor aggressiveness (Figs. 3, Table I).

Supernumerary centrioles and excess PCM in breast carcinomas and tumor derived cell lines. Analysis at the electron microscope established the structure of centrosomes in these tumors and cell lines (Fig. 4). Centrosomes containing two centrioles and nominal PCM were characteristic of epithelial cells of normal breast tissue and diploid tumors,

and also of the normal human mammary epithelial (HMEC) and MCF-7 cell lines (Figs. 4 a-b and e-f). In contrast, centrosome defects, characterized by centriole over-duplication and/or excess PCM, were observed in aneuploid breast tumors and in the aneuploid cell lines MDA-MB231 and MDA-MB435 (Figs. 4 c-d and g-h). These studies confirm the fluorescence microscopy observations reported above where diploid and near-diploid breast tissues and cell lines displayed normal centrosome structure and function, whereas aneuploid tumors show gross structural alterations in amplified centrosomes. We reported earlier that breast tumors with excess PCM show a higher number of abnormal mitoses (20).

DISCUSSION

Cancer progression occurs through accumulation of genetic alterations, cancer cell heterogeneity and, ultimately, the development of more aggressive phenotypes. Since established prognostic parameters such as tumor size, lymph node status, histologic grade and hormone receptor status do not precisely predict outcome, there is a need for new prognostic markers with increased predictive value. DNA ploidy reflects an aspect of genomic instability and is often associated with grade of differentiation, ER and p53 status and ErbB2 over-expression (32-34). Diploid tumors are generally less aggressive and have a more favorable outcome than aneuploid tumors (35). The precise control of centrosome duplication during cell cycle progression ensures equal chromosome segregation, which is critical in maintenance of diploid status. Positive and negative cell cycle regulators, such as Cdk2/cyclin E, p53, Rb, BRCA1, BRCA2 and p21/*Waf1*, are also involved in the control of centrosome duplication (17, 18, 36-41). They are likely the molecular targets linking deregulation of cell cycle checkpoints with centrosome amplification and development of chromosomal instability during tumor development and progression (42). Our studies demonstrate that breast carcinomas and tumor-derived cell lines with distinct phenotypes show different levels of centrosome amplification that reflect chromosomal instability and metastatic potential. These relationships may be common for solid tumors in general since others have drawn similar conclusions for prostate, pancreatic and colorectal cancer (8, 43, 44). Therefore, centrosome

amplification might also reflect consequent development of clonal heterogeneity for solid tumors in general, and thereby their potential for developing growth advantage and chemoresistance. Although centrosome amplification and aneuploidy can be present in breast tumors with an ER positive and wild type p53 phenotype, tumors that lose ER and/or p53 function display increased centrosome amplification and aneuploidy. These observations suggest that inactivation of p53 and loss of ER during tumor progression could result in an acceleration of chromosomal instability by uncoupling centrosome duplication from the cell cycle. Selection from the resulting heterogeneous cell population can promote more aggressive phenotypes and chemoresistance. Our results further suggest that multiple pathways can lead to centrosome amplification with subsequent chromosomal instability and tumor development. As indicated by our studies in tumor cell lines, these pathways could include over-expression of growth factor receptors and/or inactivation of tumor suppressor genes controlling cell cycle checkpoints leading to polyploidization and deregulation of centrosome duplication.

These observations suggest that centrosome amplification might be useful in monitoring chromosomal instability and in turn phenotypic diversity during tumor progression in breast cancer. For example, based on the status of centrosome phenotype, hormone responsive and lymph node negative patients may be stratified into two groups, those that require only endocrine treatment and those that require more aggressive treatment due to increased chromosomal instability associated with centrosome amplification. In conclusion we believe that associated with other established prognostic factors, centrosome amplification may be helpful in predicting outcomes and survival of patients with breast cancer.

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Table I.

Characteristics of Breast Cancer Cell Lines

	MCF-7	T-47D	MDA-231	MDA-435
Centrosome Phenotype	Normal	Amplified	Amplified	Amplified
MT Index	1.8+/- 0.6	2.9+/- 0.3	3.0 +/- 0.3	4.3 +/- 0.3
Aberrant Mitosis	10%	14%	22%	15%
Excess Centrioles	14%	38%	56%	40%
Metastatic Potential	Low	Low	High	High
ER Status	ER+	ER+	ER-	ER-
p53	Wild-type	Mutant	Mutant	Mutant

Characteristics of Breast Tumors

	Normal Tissue	Diploid Tumor	Aneuploid Tumor
Centrosome Phenotype	Normal	Normal	Amplified
Spindle Morphology	Bipolar	Bipolar	Aberrant
Lymph Node Positive	ND	0/3	6/18
ER Positive Status	5/5	3/3	12/17
p53 Wild-Type Status	5/5	3/3	10/16

FIGURE LEGENDS

Figure 1. Centrosome amplification in human breast tumor cell lines. Each column illustrates characteristics from the cell line indicated at the top of the figure: MCF-7, T-47D, MDA-231, MDA-435. The cell lines were ordered with an increasing level of centrosome amplification from left to right. Row (a): centriole number determined by indirect immunofluorescence for centrin. Normal centrosomes have two or four centrioles, while amplified centrosomes have multiple centrin spots. Row (b): pericentriolar material determined by indirect immunofluorescence for pericentrin. Normal centrosomes show staining surrounding the two centrioles, while amplified centrosomes show excess accumulation of pericentrin. Row (c): microtubule nucleation (green fluorescence) in detergent extracted cells. Nuclei were stained blue with Hoechst. Centrosome amplification results in an increased microtubule nucleating capacity. Row (d): mitotic spindle morphology. Centrioles were stained by indirect immunofluorescence for centrin (green/yellow), and mitotic motor protein HsEg5 was stained red using antibody M4-F. Normal bipolar spindles show two centrin staining spots at each pole as illustrated for MCF-7. Increased frequency of aberrant spindle morphology is seen in tumors with amplified centrosomes. Row (e): p53 localization determined by indirect immunofluorescence. Wild-type p53 in MCF-7 cells was not detected, whereas mutant p53 accumulates in nuclei of T-47D, MDA-MB231 and MDA-MB435 cells. Row (f): p21/*Waf1* localization determined by indirect immunofluorescence. p21/*Waf1* expression is highest in ER positive cells MCF-7 and T-47D, and low or not detectable in MDA-MB231 and MDA-MB435, respectively. Bars = 0.5 μm (a, b); 5 μm (c, d); and 20 μm (e, f).

Figure 2. Western blot analysis of ER, EGFR, ErbB2, p53 status, and p21/*Waf1* expression. Fifty micrograms of total protein was run in each lane. β-actin loading control is shown at the bottom of the figure.

Figure 3. Centrosome amplification and chromosomal instability in human breast tumors. (a-e): Centrin staining in green, pericentrin staining in red and co-localization in yellow. Nuclei stained blue with Hoechst stain for DNA. The tumor specimens were

selected and ordered with an increasing level of centrosome amplification from (a) to (e). ER, p53 and lymph node status and metastasis is indicated above. The tumor indicated in (a) is diploid, ER positive and p53 wild-type. The other tumors show a increased chromosomal instability and increased centrosome amplification (from left to right). (a): An ER positive, p53 wild-type, lymph node negative, diploid breast tumor showing normal levels of centrin and pericentrin staining. (b): ER positive, p53 wild-type, lymph node negative, aneuploid breast tumor showing elevated centrin staining and normal pericentrin staining. (c): ER positive, p53 mutant, lymph node negative, aneuploid breast tumor showing elevated centrin staining and normal pericentrin staining. (d): ER negative, p53 wild-type, lymph node positive, aneuploid breast tumor showing elevated staining for both centrin and pericentrin. (e): ER negative, p53 mutant, metastatic, aneuploid breast tumor showing a high level of centrin and pericentrin staining. These same tumors were also analyzed for chromosomal instability using FISH analysis for chromosomes 3 (red), 7 (green) and 17 (blue) are indicated showing gains (above the line) and losses (below the line). Bars = 10 μm.

Figure 4. Electron microscopy of centrosomes from normal and tumor tissues and breast cell lines. Normal centrosomes with two centrioles and nominal pericentriolar material are present in normal breast epithelial tissue (a), diploid tumors (b), the normal human mammary epithelial cell line, HMEC (e), and in MCF-7 cells (f). Examples of supernumerary centrioles are present in breast tumors (c) and MDA-MB231 cells (g), while some tumors and MDA-MB435 cells also show excess pericentriolar material (d) and (h), respectively. Arrowheads in (g) indicate procentrioles. Bar = 0.5 μm.

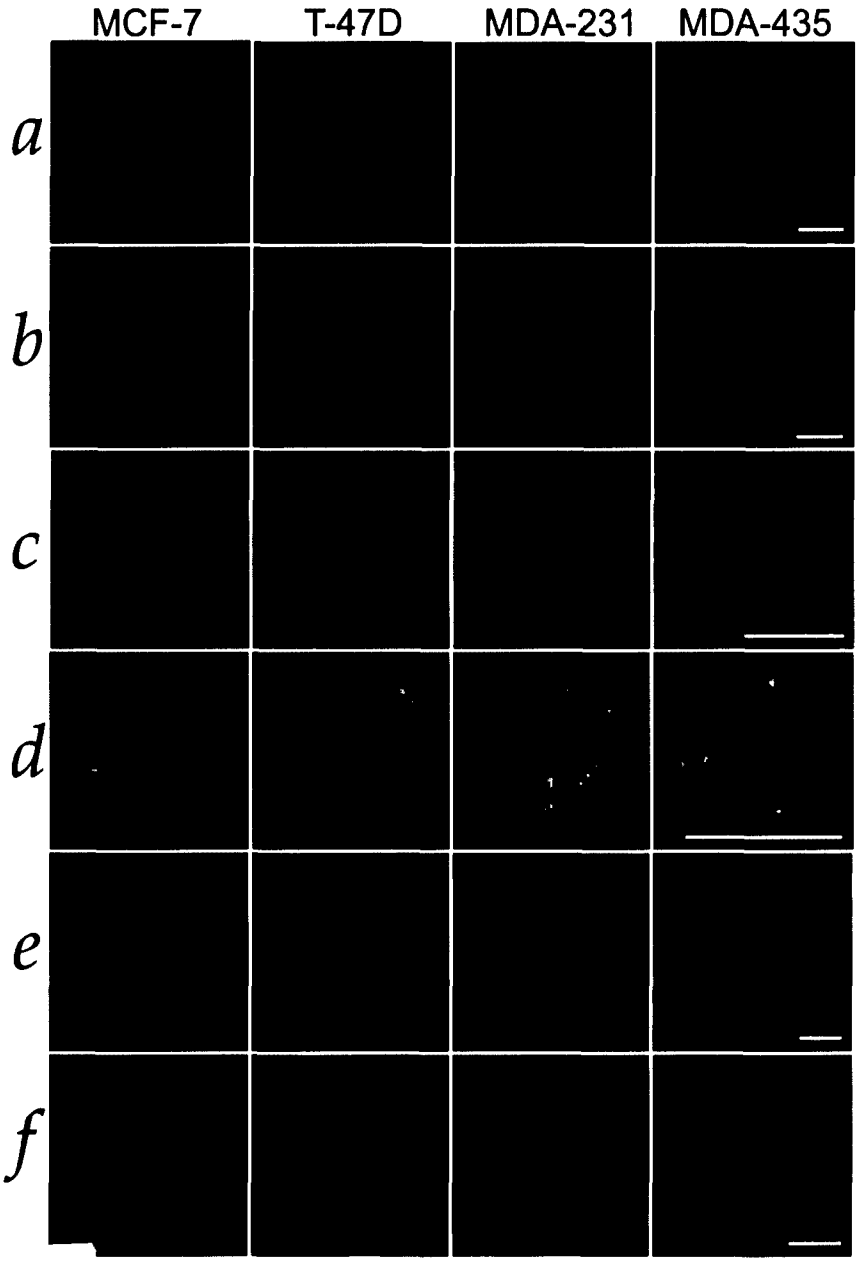
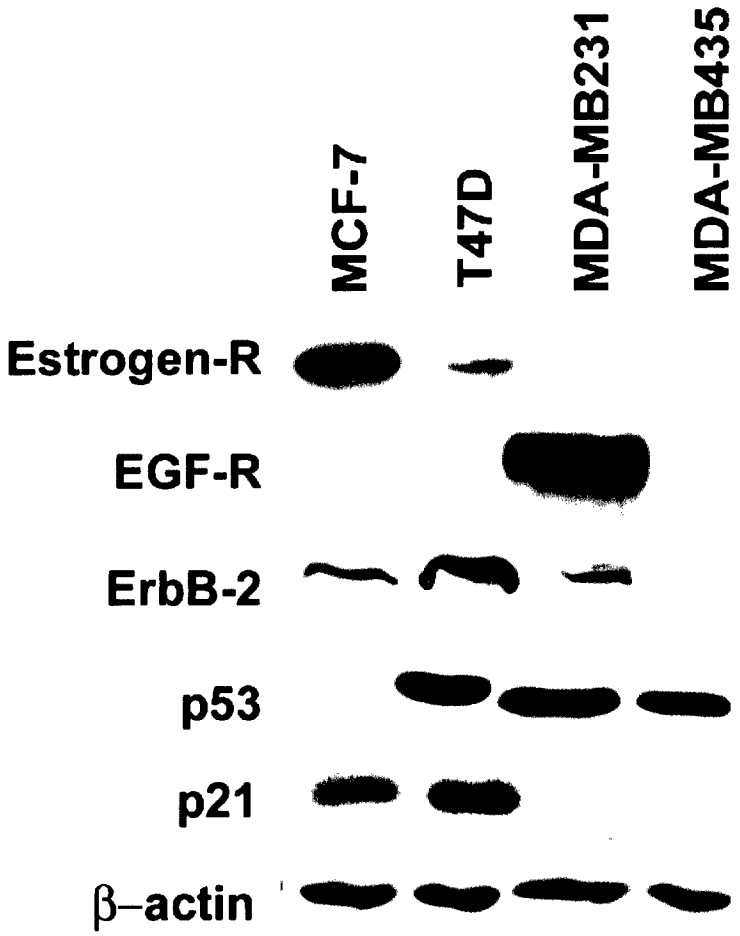


Figure 1


MCF-7
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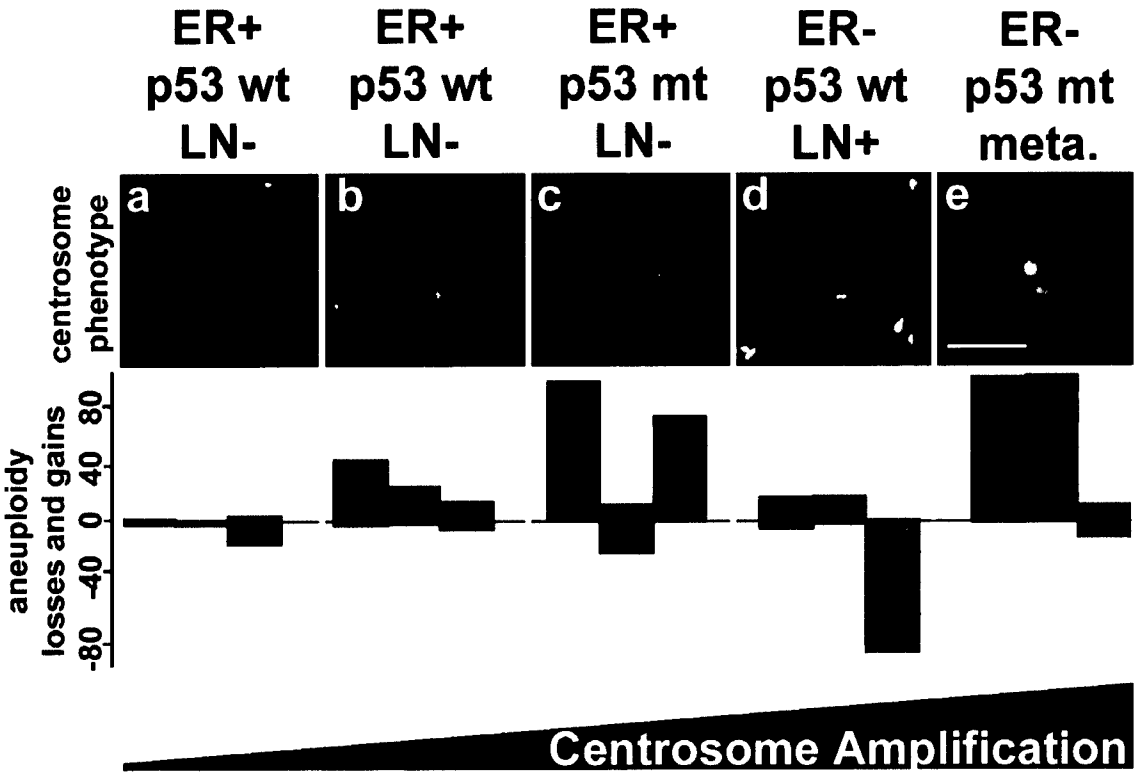
ErbB-2
p53
p21
β–actin

Figure 2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centrosome phenotype
a
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aneuploidy losses and gains
80
40
0
-40
-80
Centrosome Amplification

Figure 3

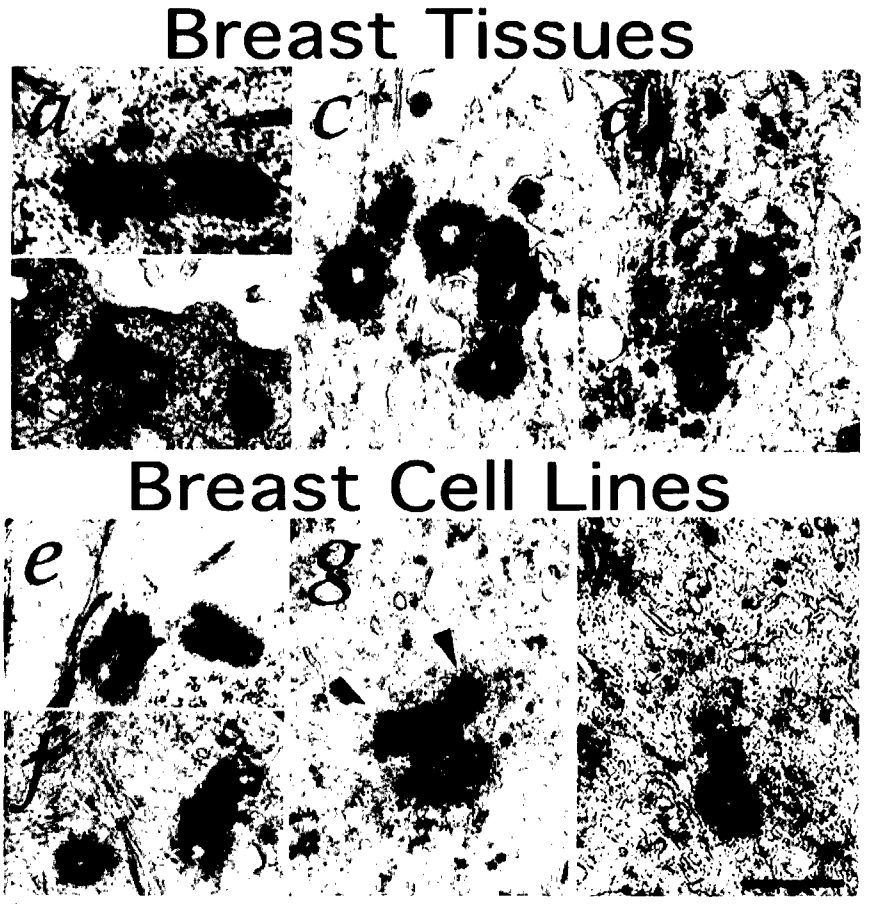

Figure 4

Appendix 2

Centrosome Amplification Correlates with Chromosomal Instability in Human Breast Tumors

Jeffrey Salisbury, Antonino D'Assoro, Clark Whitehead, and Wilma Lingle

Abstract

Approximately 80% of invasive breast tumors are aneuploid, and approximately 80% of invasive breast tumors have amplified centrosomes. In the present study, we investigated a possible relationship between centrosome amplification and aneuploidy in breast tumors. Twenty one invasive breast tumors and 7 normal breast tissues were analyzed by fluorescence in situ hybridization with centromeric probes to chromosomes 3, 7, and 17. We analyzed the tumors for both aneuploidy and unstable karyotypes as determined by chromosomal instability. These results were then tested for possible correlation with three measures of centrosome amplification – centrosome size, centrosome number, and centrosome microtubule nucleation capacity. Both centrosome size and centrosome number showed positive, significant, linear correlation with both aneuploidy and chromosomal instability. However, microtubule nucleation capacity showed no such correlation. Interestingly, microtubule nucleation capacity did correlate with loss of tissue differentiation. Together, these results indicate that different aspects of centrosome amplification have different effects on tumor development. Centrosome size and number affect chromosomal instability, while microtubule nucleation capacity affects cell, and therefore, tissue architecture. Chromosome instability has profound implications for tumor progression, while changes in cell architecture can increase metastatic potential. These results clearly demonstrate the importance of centrosome structure and function on tumor progression and reinforce the concept of the centrosome as a target for cancer therapies.

DEPARTMENT OF THE ARMY
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